

**NASA CONTRACTOR
REPORT**

NASA CR-192568

**MICROBIOLOGICAL TEST RESULTS USING THREE URINE
PRETREATMENT REGIMES WITH 316L STAINLESS STEEL**

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July 1993

(NASA-CR-192568) MICROBIOLOGICAL
TEST RESULTS USING THREE URINE
PRETREATMENT REGIMES WITH 316L
STAINLESS STEEL (Sverdrup
Technology) 19 p

N94-10360

Unclass

Interim Report

G3/51 0177409

**Prepared for
NASA-Marshall Space Flight Center
Marshall Space Flight Center, Alabama 35812**

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INTRODUCTION

The Vapor Compression Distillation (VCD) subsystem, used in the Water Recovery Test (WRT) performed at NASA/MSFC for the Environmental Control and Life Support Systems (ECLSS) of Space Station Freedom, is intended to process urine for crew reuse. Before being pumped into the subsystem, the raw urine is pretreated using a commercially available oxidant, Oxone (Dupont), and sulfuric acid (to reduce ammonia).

Previous studies in the laboratory have indicated the ability of the spore-forming bacterium, Bacillus, and fungi to survive this pretreatment process as well as an alternative treatment of sodium hypochlorite and sulfuric acid (1). Although relatively low numbers of these microorganisms have been isolated from product water downstream during the WRT, and should pose little risk to crew health, it is difficult to assess their impact on component and material failures within the VCD assembly. The presence of microbial contamination may be indicative of biofilm formation, which can restrict water flow through filters and piping, provide a means to reinoculate the system, and potentially increase corrosion rates. In the VCD subsystem and surrounding piping, which is exposed to a harsh chemical pretreatment process, corrosion may be initiated by the chemical constituents, and the microorganisms, which require time to become established within the area, may then accelerate the process.

The objective of this study was to examine the microbiological and corrosive effects of three different pretreatment protocols using 316L stainless steel, a candidate material for the water recovery system of Space Station Freedom. This paper details the microbiological results of the study.

MATERIALS AND METHODS

Approximately 7 liters of raw urine were collected for the study. Aliquots of 1600 ml were distributed into four polypropylene containers. The first container was treated with sulfuric acid and Oxone, the second with sulfuric acid and sodium hypochlorite, and the third with sulfuric acid only (in preparation for the addition of ozone). The fourth container received no pretreatment and served as a control. Table 1 lists the concentrations of the pretreatment reagents used.

Each container was inoculated with two microorganisms previously recovered from the VCD subsystem, Bacillus insolitus, a spore-forming bacterium, and a filamentous mold. Approximately 10⁷ bacteria and fungal spores were added to each aliquot. After mixing, two 800 ml volumes were removed from each container and added to each of two glass corrosion cells (EG&G PARC), one of which contained a 316L base metal specimen, and the other a welded counterpart. At the end of the test, these specimens were analyzed for biofilm development by scanning electron microscopy (SEM). Each corrosion cell contained a needle sample port to

reduce extraneous microbial contamination during sampling. All corrosion cells were continuously mixed on a stirring plate using a teflon stirring bar.

Ozone was produced by the flow of compressed oxygen through an ultraviolet light source (185 nm). An ozone concentration in air of approximately 32 mg/hr was determined based on the results of similar laboratory tests using Drager tubes and an oxygen flow rate of 0.5 standard cubic feet per hour (scfh). The ozone produced was split and bubbled into each acid-treated sample using a fritted glass sparger (EG&G PARC). Ozonation of the corrosion cells was terminated after 3 days of continuous operation. Residual ozone concentrations were measured by the iodometric method (2), which is less accurate than the indigo dye method, but less susceptible to colorimetric interferences.

Test parameters examined included pH, macroscopic observation (color, turbidity and odor) and microbial counts of the solutions and surfaces (SEM). The pH was measured using standard pH paper (0.5 increments). Changes in color and turbidity were documented by photographic records.

Microbiological analysis of the solutions was performed by collecting approximately 2 ml of sample from the sample port using sterile evacuated tubes. The sample was diluted as necessary in sterile phosphate buffered saline (PBS), and spread plated onto R2A agar. The plates were incubated at 28C for 5 days. After enumeration, bacterial colonies exhibiting different colonial morphologies were subcultured to brain heart infusion agar in preparation for identification. The Minitek Identification System (BBL) and Biolog Identification System (Biolog) were employed for identification of bacterial isolates.

At the end of the test (21 days), base and welded metal specimens were prepared for SEM analysis by immersion in sterile formaldehyde (3.7%) for 2 minutes, followed by drying in absolute alcohol and freon for 2 minutes each. Fixed samples were sputter-coated with Au-Pb at 30-35mA for 30 seconds. Coupons were observed under a scanning electron microscope (Hitachi) with a field emission source (Quantum).

RESULTS

Macroscopic Observations

Initial ozonation of the sulfuric acid treated corrosion cells resulted in the production of excessive bubbles and required temporary discontinuation of the ozonation process. After approximately 10 hours, the ozonator was restarted and allowed to run for 48 hours without interruption. After 1 day of ozonation, the corrosion cell containing the welded metal specimen was less colored than the base metal counterpart (figure 1) and contained a residual ozone concentration approximately 2-fold higher than in the base metal counterpart. This difference likely resulted

from unequal air flow distribution and/or varied sparger mixing efficiency at the gas-water interface. A comparison of the three pretreatments and control corrosion cells at this time is shown in Figure 2 (top). The corrosion cells receiving the hypochlorite pretreatment and the control corrosion cells became increasingly turbid. The Oxone pretreatment cells were discolored but generally free of turbidity. Corrosion cells receiving ozonation had the least color and turbidity. As the test progressed, ozonated corrosion cells decreased slightly in color and remained free of turbidity, followed by Oxone-treated corrosion cells, hypochlorite-treated corrosion cells, and untreated controls. Figure 2 (bottom) shows a comparison of the three pretreatments after 21 days.

Microbiological Enumeration and Identification

All pretreatments significantly reduced microbial numbers versus controls in both base and welded metal sample solutions (figures 3a and 3b, respectively). Microbial levels in the ozonated base metal corrosion cell remained lower than in the other pretreatments during the test, maintaining a relatively stable concentration of approximately 1×10^2 cfu/ml. The Oxone and sodium hypochlorite pretreatments resulted in roughly the same number of microbes, decreasing from approximately 1×10^5 to 1×10^3 cfu/ml during the test. For the welded metal samples, ozone again maintained a stable microbial population of less than 10 cfu/ml. This microbial count was approximately two orders of magnitude lower than that of the ozone base metal counterpart and three orders of magnitude less than that of the other pretreatments containing the welded metal. As noted previously, this sample also contained a higher residual ozone concentration.

Neither of the two microorganisms inoculated into the corrosion cells was recovered from the control samples. However, two other species, Pseudomonas fluor/putida, a rod-shaped bacterium, and Streptococcus faecium, a coccal form, were isolated. In contrast, only inoculated organisms were found in the pretreatment samples. For the Oxone samples, both the bacillus and filamentous mold were isolated early in the study whereas only the bacillus was found after approximately 120 hrs. Bacillus sp. was the only organism isolated from ozonated samples.

Scanning Electron Microscopy

Figure 4(a-h) shows photomicrographs of various metal specimen surfaces. Figure 4a shows the ozone treated sample which is largely devoid of bacteria. The ozone welded sample was similar in appearance. Figure 4b represents the partially melted region of the welded coupon treated with hypochlorite. At a higher magnification, the localization of primarily coccal bacterial types can be seen in the remelted grain boundaries (Figure 4c). Very few bacteria were found on the corresponding base metal surface (Figure 4d). Similarly, the Oxone treated base metal contained relatively few bacteria randomly distributed over the

surface (Figure 4e), whereas localized attachment was observed near heat-affected regions of the welded sample (Figure 4f). At higher magnification, an extensive biofilm can be observed (figure 4g). Control base and welded samples contained the greatest populations of bacteria, consisting of both rod and coccal bacterial types (figure 4h).

DISCUSSION

Due to unequal distribution and/or mixing of ozone between the corrosion cells, the welded sample solution was significantly lower in microbial numbers than its base metal counterpart, with an average of 2 cfu/ml compared to 200 cfu/ml for the base metal sample in the ozone pretreatment. The Oxone and hypochlorite pretreatments were similar in their ability to reduce microbial numbers in fluids, but were less effective than ozone. The two inoculated organisms were recovered from the various pretreatments, much as they were recovered during sampling of the VCD subsystem. In the control samples, the absence of a pretreatment afforded other species present in the urine the opportunity to outcompete the inoculated organisms.

The surfaces of all pretreatment base metal samples remained relatively free of bacteria; however, welded samples from both the Oxone and hypochlorite pretreatments contained bacteria in heat-affected zones of the metal. Other investigators have noted the apparent preferential attachment of bacteria at or near regions of surface heterogeneities, such as weldment sites, although the mechanism is not completely understood (3,4,5).

Although the predominant morphology found on the metal surfaces was coccal, rod-shaped bacteria were also observed. Both rod and coccal forms were also found in the liquid from the control samples. However, microbiological analysis of liquid from the pretreatment samples revealed only rod-shaped bacteria (Bacillus sp.). This result is similar to what has been observed during Phase III testing of the MSFC ECLSS Water Recovery System (6). Rod-shaped bacteria such as Pseudomonas spp., Klebsiella pneumoniae and Bacillus spp. were the predominant morphological types isolated from the urine pretreatment system. However, the predominant morphological type isolated in the product tanks downstream of this subsystem was coccal. It is conceivable that the coccal forms preferentially colonized the surface of the VCD subsystem, as they did the metal specimens here, and developed biofilms which provided protection from the pretreatment processes and attempts at isolation from the liquid. Portions of the protective biofilm containing the bacteria may have eventually sloughed off and colonized downstream of the subsystem.

Because fungi were isolated early in this test from only Oxone and hypochlorite samples and were not seen by surface SEM analysis, it appears that all pretreatments eventually removed these organisms. However, these organisms have been isolated from urine processing assemblies during operational tests (1,7). This

suggests that a longer contact time and/or increased chemical concentration may be required to remove these organisms if Oxone or hypochlorite pretreatments are used. The control of these microorganisms should be considered especially important in an environment such as the VCD, where microbial diversity appears to be somewhat low. In such an environment, the rate of production of metabolic acid by-products by these organisms may be greater than that which can be utilized by other organisms present, thereby altering the pH of the surrounding metal and increasing the corrosion potential.

SUMMARY

The pH of all corrosion cells receiving pretreatment remained stable at approximately 3.0. Control pH values increased from 6.0 to 8.5 during the test. Corrosion cells receiving ozone pretreatment showed a considerable reduction in turbidity and color compared to the other pretreatments and control samples.

All three of the urine pretreatment regimes significantly reduced microbial numbers versus controls. Of the three, ozone was significantly more effective than the other two. Hypochlorite and Oxone pretreatments reduced microbial numbers equally. Neither of the inoculated microorganisms, a filamentous mold and a rod-shaped spore forming bacterium, Bacillus insolitus was isolated from either of the control corrosion cells. The two species isolated, Pseudomonas fluor/putida, a rod-shaped bacterium, and a coccus, Streptococcus faecium, appeared better suited to this environment. In contrast, the urine pretreatment regimes selected for the inoculated microbes, which had been recovered from the VCD subsystem environment. Both Bacillus sp. and the mold were isolated in the Oxone and hypochlorite samples early in the study, with only the bacillus found after day 4 of testing. Bacillus was the only species identified from samples treated with ozone.

Base metal specimens from the various pretreatments were relatively free of bacterial attachment, with no defined biofilms observed. However, well-developed biofilms were noted in heat-affected regions of welded specimens from both the hypochlorite and Oxone pretreatments. Few bacteria were observed in the same regions of the ozone pretreatment sample. Coccal forms predominated on the surface of all metal specimens, indicating that while the two inoculated organisms were present in the liquid, other species appeared to colonize the surfaces. Additional studies are necessary to confirm this observation.

CONCLUSIONS

In the determination of effective biocide treatment for space systems, special consideration must be given to factors such as biocide storage, methods of administration and removal, and material compatibility. Of equal importance is the efficacy of

the biocide chosen. Experience on Earth has shown that inadequate disinfection schemes result in microbial adaptation and resistance, largely due to the presence of surface biofilms. Thus, a beneficial biocide is one that maintains both minimal liquid and surface microbial contamination. The results of this test indicate that ozone was the most effective of the three pretreatments in satisfying these requirements.

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Table 1

Pretreatment Reagents and Concentrations Based on
1 liter Volume of Raw Urine

Oxone	5.0g	H ₂ SO ₄	2.32g
Sodium Hypochlorite (5%)	4.0ml	H ₂ SO ₄	2.32g
Ozone	32.0mg/hr	H ₂ SO ₄	2.32g

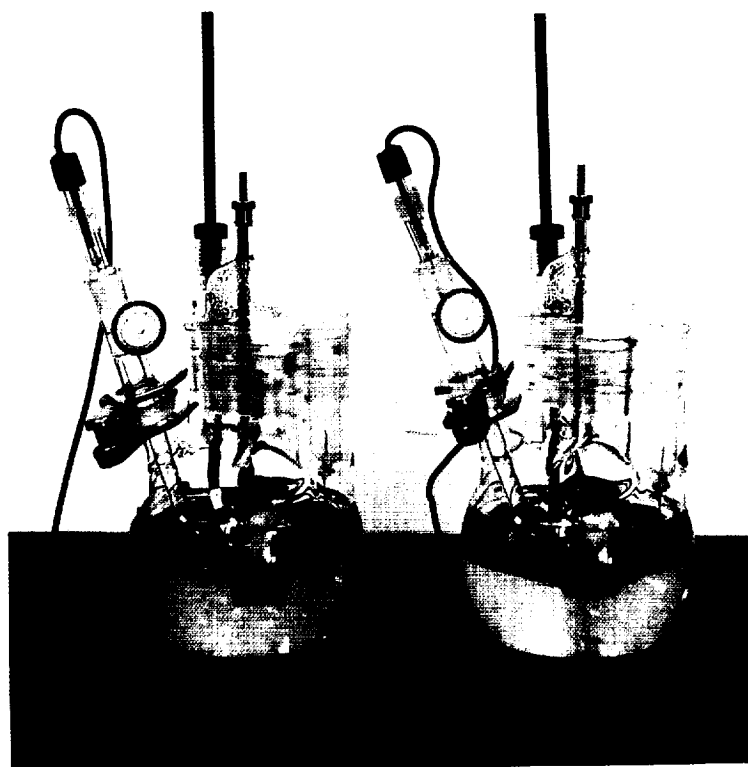


Figure 1. Comparison of ozone base (left) versus welded metal (right) corrosion cells after day 1.

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COLOR PHOTOGRAPH

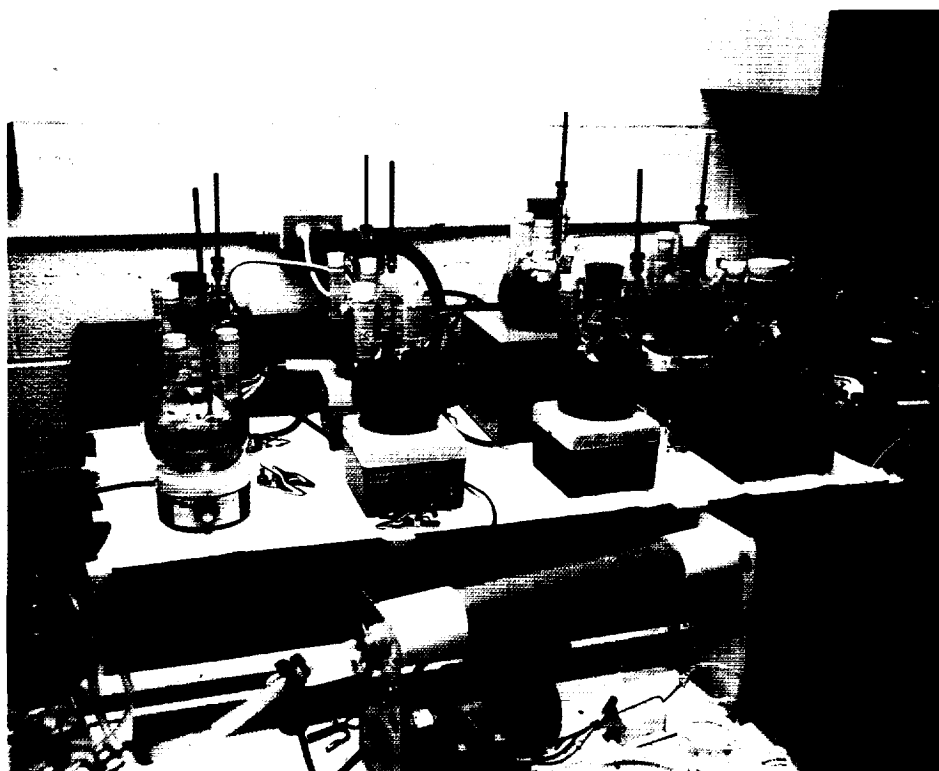
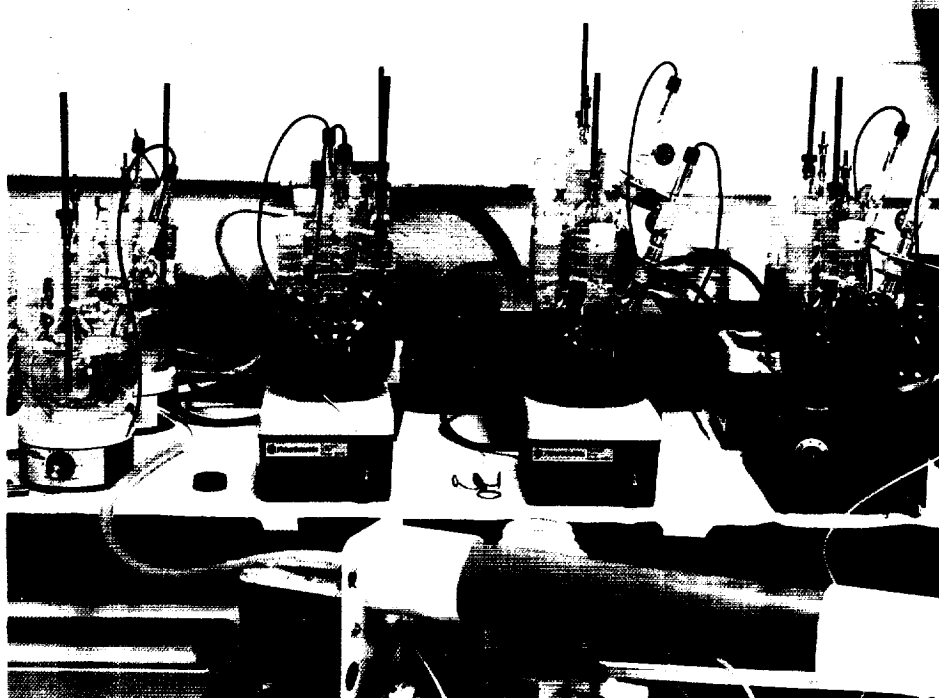


Figure 2. Changes in color and turbidity over a 3-week period. (top) Corrosion cells after day 1 (left to right, ozone, control, hypochlorite, Oxone). (bottom) Corrosion cells after 21 days (left to right, ozone, hypochlorite, Oxone, control).

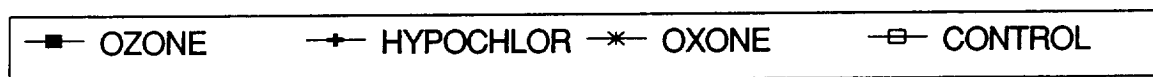
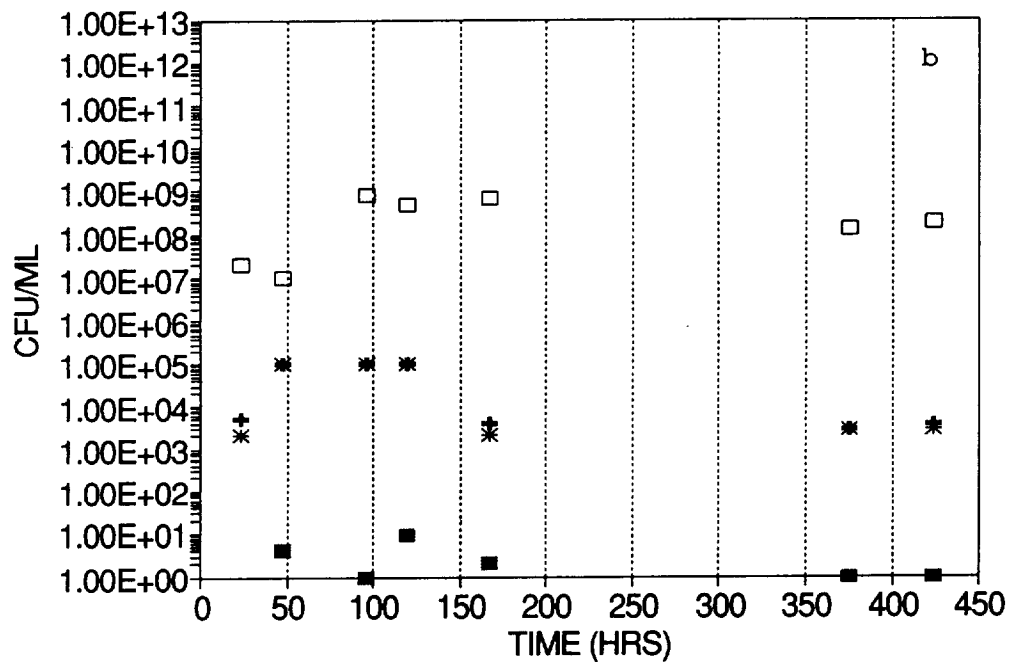
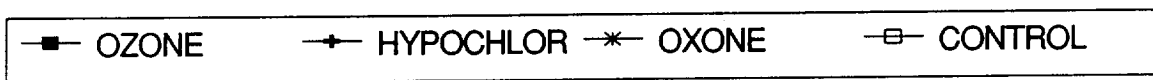
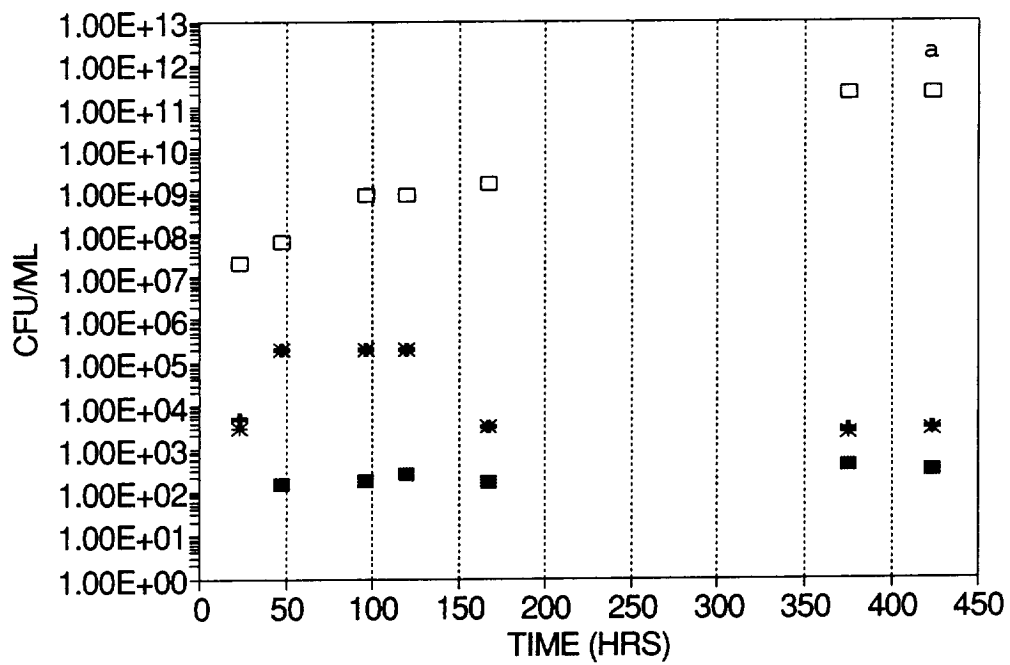


Figure 3. Changes in microbial numbers in control and pretreatment corrosion cells over a 3-week period.
 (a) Base metal samples and (b) welded metal samples.

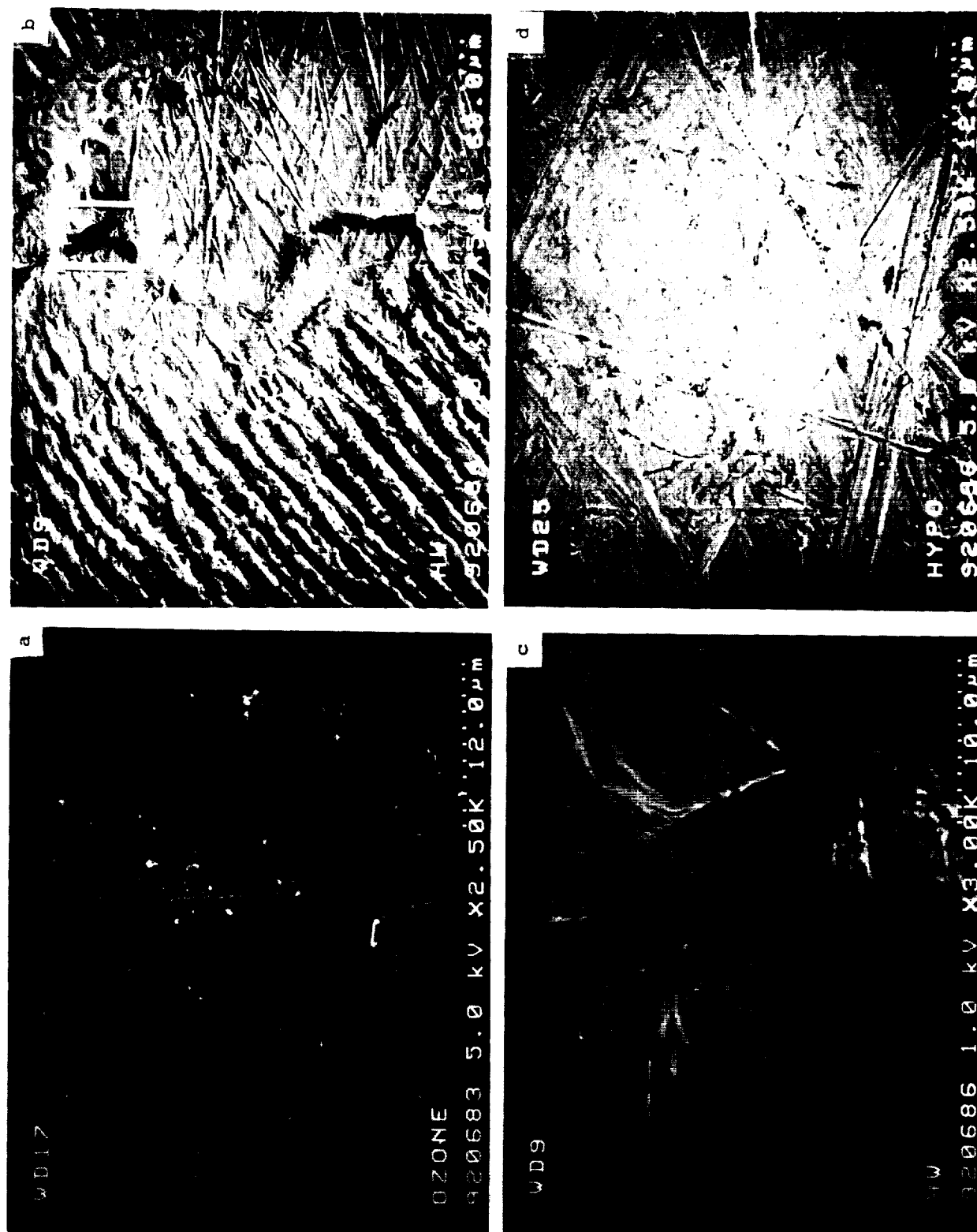


Figure 4. SEM photomicrographs of 316L specimens exposed to urine and urine pretreatments. (a) ozone treated, (b) hypochlorite weld, (c) region b, higher magnification, and (d) hypochlorite base.

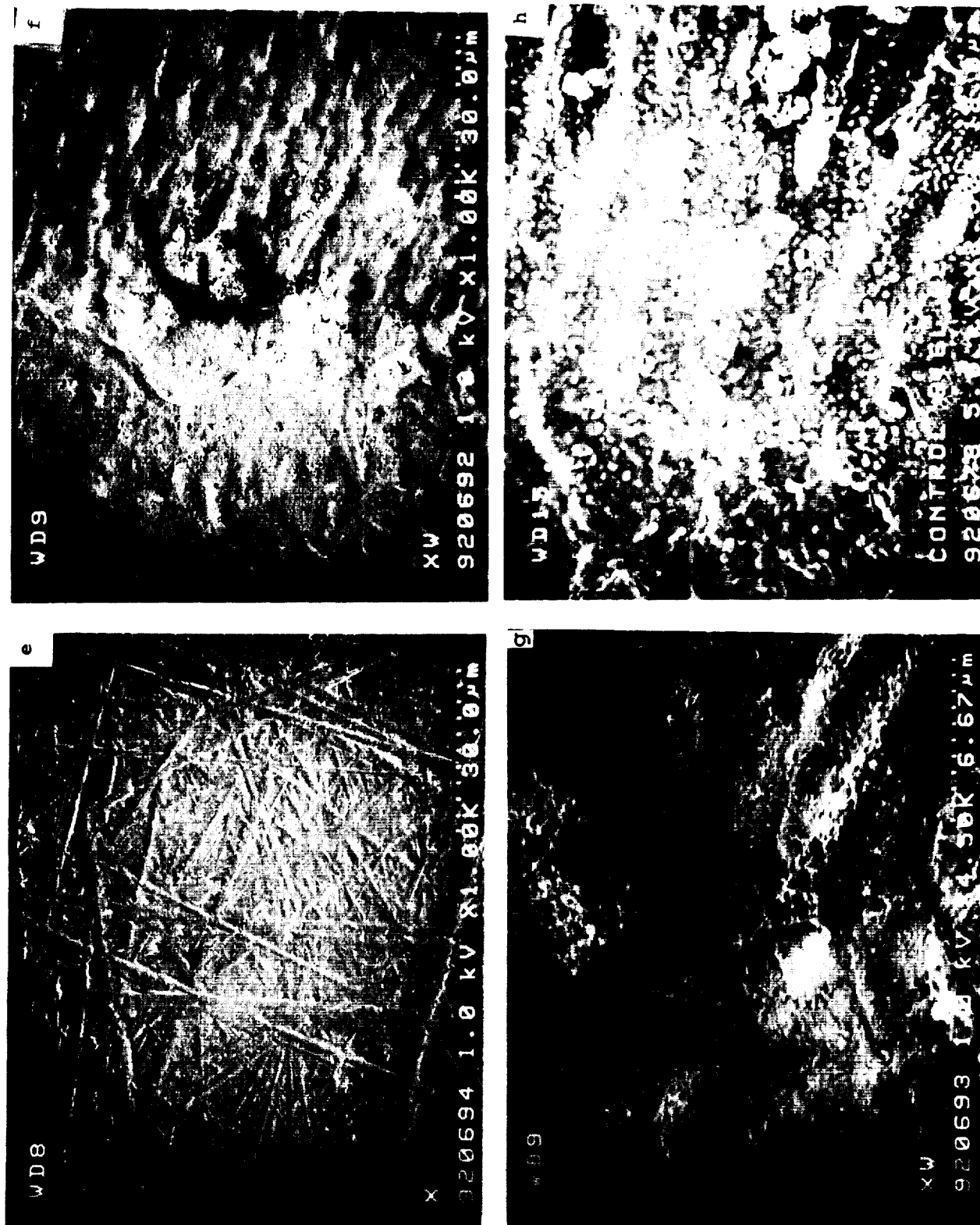


Figure 4. SEM photomicrographs of 316L specimens exposed to urine and urine pretreatments (continued). (e) Oxone, (f) Oxone weld, (g) region f, higher magnification, and (h) control weld.

APPROVAL

MICROBIOLOGICAL TEST RESULTS USING THREE
URINE PRETREATMENT REGIMES WITH 316L
STAINLESS STEEL

By Timothy L. Huff

The information in this report has been reviewed for technical content. Review of any information concerning Department of Defense or nuclear energy activities or programs has been made by the MSFC Security Classification Officer. This report, in its entirety, has been determined to be unclassified.



Paul H. Schuerer
Director, Materials and Processes Laboratory

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 1993	3. REPORT TYPE AND DATES COVERED Contractor Report		
4. TITLE AND SUBTITLE Microbiological Test Results Using Three Urine Pretreatment Regimes With 316L Stainless Steel		5. FUNDING NUMBERS Contract NAS8-37814		
6. AUTHOR(S) T.L. Huff		8. PERFORMING ORGANIZATION REPORT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Sverdrup Technology, Inc. Huntsville, Alabama 35806		10. SPONSORING / MONITORING AGENCY REPORT NUMBER NASA CR-192568		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) George C. Marshall Space Flight Center Marshall Space Flight Center, Alabama 35812		11. SUPPLEMENTARY NOTES Technical Monitor: Dr. Elizabeth Rodgers Materials and Processes Laboratory, Science and Engineering Directorate George C. Marshall Space Flight Center, MSFC, Alabama 35812		
12a. DISTRIBUTION / AVAILABILITY STATEMENT Unclassified—Unlimited		12b. DISTRIBUTION CODE		
13. ABSTRACT (Maximum 200 words) Three urine pretreatments, (1) Oxone (Dupont) and sulfuric acid, (2) sodium hypochlorite and sulfuric acid, and (3) ozone, were studied for their ability to reduce microbial levels in urine and minimize surface attachment to 316L stainless steel coupons. Urine samples inoculated with <i>Bacillus insolitus</i> and a filamentous mold, organisms previously recovered from the vapor compression distillation subsystem of NASA Space Station <i>Freedom</i> water recovery test were tested in glass corrosion cells containing base or weld metal coupons. Microbial levels, changes in pH, color, turbidity, and odor of the fluid were monitored over the course of the 21-day test. Specimen surfaces were examined by scanning electron microscopy at completion of the test for microbial attachment. Ozonated urine samples were less turbid and had lower microbial levels than controls or samples receiving other pretreatments. Base metal coupons receiving pretreatment were relatively free of attached bacteria. However, well-developed biofilms were found in the heat-affected regions of welded coupons receiving Oxone and hypochlorite pretreatments. Few bacteria were observed in the same regions of the ozone pretreatment sample.				
14. SUBJECT TERMS water reclamation, disinfection, contamination, space station, ozone, Oxone, chlorine, urine, bacteria, fungi, microbiology, biofilm 316L stainless steel		15. NUMBER OF PAGES 19		16. PRICE CODE NTIS
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

